In vitro regulation of Mac-1 expression on bovine polymorphonuclear leukocytes by endotoxin and tumor necrosis factor- α at different stages of lactation

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Abstract

The purpose of this in vitro study is to clarify some of the underlying mechanisms leading to the decreased migratory capacity of polymorphonuclear leukocytes (PMN) during mastitis in dairy cows soon after calving. Surface expression of Mac-1 (CD11b, CR3) on PMN and of CD14 on monocytes was measured in early- (EL), peak- (PL), and midlactation (ML) by flow cytometric analysis. In addition, we evaluated the effect of lipopolysaccharide (LPS) and tumor necrosis factor (TNF)- α on CD11b surface expression in PMN at different stages of lactation in a whole blood model. During EL, while resting monocytes expressed diminished levels of CD14, the basal expression of CD11b on PMN was not significantly altered. The relative increase of CD11b on PMN after incubation with LPS or TNF- α did not significantly differ among EL, PL, or ML at any of the concentrations tested. The current findings do not support an important role for basal CD11b levels nor for a defective mobilization of CD11b by LPS and TNF- α in the reduced migratory capacity of PMN during EL.

Résumé

Cette étude in vitro se propose de clarifier certains mécanismes fondamentaux conduisant à la diminution de la capacité de migration des PMN chez la vache laitière pendant les mammites juste après le vêlage. L'expression en surface des récepteurs Mac-1 (CD11b, CR3) sur des PMN et du CD14 sur des monocytes a été mesurée au début (EL), au pic (PL) et à la moitié de la lactation (ML) par analyse en cytométrie en flux. En outre, nous avons évalué l'effet du lipopolysaccharide (LPS) et du facteur nécrosant des tumeurs (TNF)-α sur l'expression en surface de CD11b sur des PMN pendant les différentes périodes de lactation dans un modèle de sang complet. Pendant EL l'expression basale de CD11b sur les PMN n'a pas été modifiée significativement alors que les monocytes au repos montraient une diminution des niveaux de CD14. L'augmentation relative de CD11b sur les PMN, après leur incubation avec du LPS ou du TNF-α, n'a pas différé de manière significative entre EL, PL ou ML pour aucune des concentrations testées. Ces résultats ne suggèrent pas un rôle important des niveaux basaux de CD11b ni de la mobilisation altérée de CD11b par le LPS et TNF-α sur la réduction de la capacité de migration des PMN pendant le EL.

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Periparturient cows frequently suffer from an increased severity of coliform mastitis as a consequence of their relative inability to rapidly mobilize polymorphonuclear leukocytes (PMN) into the mammary gland in response to infection (1,2). However, the mechanism by which the migratory efficiency of PMN decreases during the periparturient period is largely unknown.

Leukocyte surface glycoprotein Mac-1 (CD11b, CR3) is a member of the β_2 -integrin family and is involved in adhesion and migration of bovine PMN into the udder (3,4). Recent studies have demonstrated that an important fraction of the total CD11b content expressed by bovine PMN is stored intracellularly and is upregulated in vitro as the result of different types of stimuli (5–7). The quantitative and qualitative changes in CD11b expression at the PMN surface caused by inflammatory mediators such as lipopolysaccharides (LPS) and tumor necrosis factor (TNF)- α correlate with increased CD11b-dependent adherence and transendothelial migration (8,9). CD14 is a major endotoxin-binding molecule expressed mainly by monocytes.

Therefore, the role of LPS in mediating production of TNF- α upon binding to this receptor is particularly relevant during gram-negative infections.

Although a balanced cortisol response is required for cellular homeostasis, this hormone has also been implicated in PMN immunosupression in early lactating cows. Indeed, systemic cortisol levels increase nearly 4-fold in cattle at calving and are highest during EL (10). It has been reported that cortisol decreases β_2 -integrin expression in vivo and reduces CD11b upregulation upon in vitro stimulation with different proinflammatory mediators (5,11,12). Therefore, we evaluated basal levels of CD11b, and examined whether LPS and TNF- α influence the amount of CD11b surface protein on the peripheral PMN of dairy cows during EL, as compared to PL and ML. The role of TNF- α in the upregulation of CD11b by LPS was also studied. In addition, we examined the expression of CD14, the major LPS-binding molecule, on monocytes.

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Table I. Effect of increasing concentrations of lipopolysaccharides (LPS) with (+) or without (-) anti-bovine tumor necrosis factor (TNF)- α mAb (A) or of TNF- α (B) on the expression of CD11b (MFI) in circulating polymorphonuclear leukocytes (PMN). Data represent means \pm standard error $(s_{\bar{x}})$. No differences could be found between early- (EL), peak- (PL), and midlactation (ML). Statistically significant differences between control and samples stimulated with LPS (I) or TNF- α (II), and between control and samples preincubated with anti-TNF- α mAb (III) are indicated

A.							
LPS (ng/mL)	anti-TNF-α mAb	EL (n = 8)	PL (n = 8)	ML (n = 8)	Average $(n = 24)$	I. Control vs. LPS	III. mAb effect
0	_	28.14 ± 1.23	31.44 ± 2.08	32.03 ± 3.11	30.54 ± 1.26	_	_
0	+	28.69 ± 1.25	31.92 ± 2.02	31.34 ± 2.88	30.65 ± 1.17	_	ns
1	_	32.70 ± 1.86	36.60 ± 2.81	39.20 ± 4.30	36.16 ± 1.88	P < 0.001	_
1	+	29.76 ± 1.30	35.12 ± 2.40	34.18 ± 3.63	33.02 ± 1.51	_	P < 0.001
10 ²	_	32.63 ± 1.51	38.42 ± 2.95	40.12 ± 4.74	37.06 ± 1.90	P < 0.001	_
10 ²	+	31.39 ± 1.65	37.00 ± 2.78	36.76 ± 4.03	35.05 ± 1.66	_	P < 0.001
104	_	32.09 ± 1.85	37.42 ± 2.61	37.63 ± 4.42	35.71 ± 1.81	P < 0.001	_
104	+	30.59 ± 1.77	37.01 ± 2.70	36.51 ± 4.24	34.70 ± 1.79	_	ns
B.							
$\overline{\text{TNF-}\alpha \text{ (ng/mL)}}$	EL (n = 7)	PL (<i>n</i> = 7)	ML (n = 7)	Average $(n = 2)$	1) II. Control vs. 7	ΓΝΓ-α	
0	28.02 ± 1.22	31.61 ± 2.44	27.29 ± 2.75	28.97 ± 1.29	_		
1	31.16 ± 2.11	35.81 ± 3.23	31.37 ± 3.24	32.78 ± 1.66	P < 0.002	1	
10	33.29 ± 2.22	36.94 ± 3.08	33.40 ± 3.45	34.54 ± 1.67	P < 0.002	1	
50	35.01 ± 2.16	39.63 ± 3.15	36.24 ± 4.15	36.96 ± 1.84	P < 0.002	1	

ns - Not significant

Experiments were performed on 49 clinically healthy, Holstein-Friesian dairy cows, in their 1st to 4th lactation, from the Ghent University dairy herd (Biocentrum Agri-Vet, Melle, Belgium). Three groups of cows were sampled: 15 cows in EL (15.1 \pm 1.95 d after parturition), 15 in PL (57.7 \pm 3.62 d after parturition), and 19 in ML (133 \pm 3.63 d after parturition). Immediately after morning milking, blood was aseptically collected in pyrogen-free heparinized vacuum tubes (Chromogenix, Milano, Italy) by jugular venipuncture between 08:00 and 09:00. Smears were prepared from whole blood and stained (Hemacolor; Merck, Darmstadt, Germany). Differential microscopic counts were determined by counting 200 cells. Only samples with PMN counts greater than 90% of the total granulocyte population were included in the study.

Recombinant human TNF- α (Calbiochem, San Diego, California, USA) was diluted in phosphate buffered saline solution (Dulbecco's PBS [DPBS]; Gibco, Paisley, United Kingdom) containing 0.1% fetal calf serum (Sigma, Bornem, Belgium) and stored in aliquots at -70° C until use. Anti-bovine TNF- α monoclonal antibody (mAb) (13) was diluted in DPBS. Lipopolysaccharides (*Escherichia coli* O111:B4; Sigma-Aldrich) were dissolved in 0.9% saline.

To test the effect of the different compounds, pyrogen-free polystyrene round-bottom tubes (Becton Dickinson, San José, California, USA) were used containing 90 μ L whole blood. Samples were preincubated with 10 μ L anti-bovine TNF- α mAb for 15 min at room temperature. After the addition of LPS (1 to 10^4 ng/mL) or TNF- α (1 to 50 ng/mL), concentrations routinely used for in vitro experiments, all samples were incubated for 90 min at 37° C. Further incubation for 30 min at 37° C was done with either 50 μ L anti-bovine Mac-1 (clone CC126 mAb; ProBio, Margate, Kent, United Kingdom) or $10~\mu$ L anti-ovine CD14 (clone VMP65; Serotec, Oxford, United Kingdom) at saturating concentrations diluted in control solution, RPMI 1640 (Gibco Brl, Scotland, United Kingdom), supplemented

with 1% bovine albumin fraction V (Merck) and 0.2% NaN₃. Following incubation, indirect immunofluorescence was performed as previously described (14).

Specimens were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For each sample, 20 000 events were recorded in list mode and displayed on a logarithmic scale. Leukocyte populations were characterized by forward and side light scattering characteristics, and dot plots were gated for PMN and monocytes. The PMN population was additionally identified by CH138A mAb (VMRD, Pullman, Washington, USA), which recognizes bovine granulocytes. The mean fluorescence intensity (MFI) and percent of positive cells in these areas were calculated after plotting the fluorescein isothiocyanate (FITC) fluorescence histograms. Nonspecific background fluorescence was any fluorescence associated with leukocytes incubated with FITC-labeled secondary antibody alone.

Basal levels of CD11b and CD14 were compared for the 3 stages of lactation by analysis of variance. The MFI of CD11b was statistically processed as a function of the LPS and TNF- α concentrations using a mixed model. Cow was introduced as a random effect, while lactation stage, LPS and TNF- α concentrations, and their interactions were added as categorical fixed effects. Pairwise comparisons were adjusted by Tukey's method. This model was also fitted in the presence of anti-bovine TNF- α mAb.

CD11b density on unstimulated blood PMN was evaluated in 9 cows per stage of lactation. A slightly lower, but non-significantly altered, MFI was found during EL (28.4 \pm 1.03) as compared to PL (32.6 \pm 2.07) and ML (30.4 \pm 2.88). A similar high proportion of circulating PMN with positive staining for CD11b (~97%) was detected during the 3 stages of lactation. Decreased CD14 surface density on monocytes (P < 0.05) was found in EL (9.22 \pm 1.01) versus PL (14.68 \pm 2.12) or ML (14.26 \pm 1.38) on resting PMN as evaluated in

6 additional cows per lactational group. Following incubation with LPS or TNF- α , an increase of CD11b surface density on PMN was detected (Table I). A maximal response of 23% (P < 0.001) was already observed with the lowest LPS dose (1 ng/mL). Each of the different TNF-α concentrations tested elicited an upregulatory response of CD11b density. The observed increase ranged from 13.9% with 1 ng/mL TNF- α (P < 0.001) to 27.6% with 50 ng/mL TNF- α (P < 0.001). However, stimulation of bovine PMN with any of the LPS or TNF-α doses resulted in a similar pattern of CD11b expression during the 3 lactational periods. To determine whether CD11b upregulation could be inhibited in this whole blood assay system, cells were pre-incubated for 15 min at room temperature with antibovine TNF- α mAb. This antibody was used in an attempt to block the effect of different concentrations of recombinant human TNF- α (1, 10, and 100 ng/mL) on CD11b upregulation in 4 ML cows. Although no inhibitory effect was observed on the TNF- α -induced upregulation of CD11b (data not shown), the ability of LPS to upregulate CD11b density on circulating PMN was significantly decreased by anti-bovine TNF- α mAb (Table I).

Polymorphonuclear leukocytes from cows in EL show defects in the migratory process and are responsible for the increased susceptibility of periparturient animals to coliform mastitis. The release of immature PMN from the bovine bone marrow into the circulation (15) may potentially affect the average level of expression of CD11b during EL (7). In the current study, a whole blood model was used to investigate the possible role of altered CD11b levels in EL cows.

Significant differences could not be detected in either the MFI of CD11b nor in the percentage of CD11b⁺ cells among the 3 stages of lactation in unstimulated PMN. Following treatment with LPS or TNF- α we could show an upregulatory effect on CD11b MFI. These results are in agreement with previous studies (2,5,14,16).

The CD14 molecule is considered the major LPS binding receptor on mononuclear phagocytes and associates with Toll-like receptor 4 and MD-2 to form the functional LPS receptor complex (17). Significantly lower basal values of CD14 MFI were detected in unstimulated bovine monocytes from EL cows. The decreased level of CD14 during EL was not, however, accompanied by altered upregulation of CD11b at the PMN surface upon LPS stimulation. It has been previously reported that CD14 mainly mediates β_2 -integrin upregulation at low concentrations of LPS (<0.5~ng/mL), while at higher doses other, low-affinity, LPS binding receptors may also participate (18). Therefore, current results may be explained by the possible involvement of independently acting LPS receptors, such as moesin (19).

The observation that anti-bovine TNF- α mAb fails to inhibit CD11b upregulation by recombinant human TNF- α could be due to the inability of the antibody to cross-react between species. Nevertheless, the capacity of LPS to upregulate CD11b density on circulating PMN could be significantly suppressed by this mAb (Table I). Therefore, part of the response to LPS, in the whole blood system, seems to be mediated by the interaction between monocytes and PMN via the release of cytokines, such as TNF- α , as has been suggested before (5). In accordance, other studies have shown that stimulation of isolated bovine monocytes with LPS leads to the production of TNF- α (20,21). The comparable upregulation elicited by LPS on CD11b density among the different stages of lactation

might, thus, be alternatively explained by a compensatory effect of monocytes, given the greater potential of these cells to produce TNF- α after LPS stimulation, their decreased sensitivity to cortisol during EL, or both (21–22). Under the current experimental conditions, we also found that the pro-inflammatory mediator TNF- α could upregulate CD11b surface expression on PMN to a similar extent for each of the 3 lactation periods considered.

In conclusion, our results suggest that basal CD11b levels, a defective mobilization of CD11b from intracellular pools, or both, caused by LPS or TNF- α do not underlie the compromised ability of bovine PMN to migrate into the inflammatory site during EL.

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